Award Number: W81XWH-12-1-0582

TITLE: Down-Regulation of Olfactory Receptors in Response to Traumatic Brain Injury Promotes Risk for Alzheimer's disease

PRINCIPAL INVESTIGATOR: Giulio Maria Pasinetti MD., PhD

CONTRACTING ORGANIZATION: Icahn School of Medicine at Mount Sinai
New York, NY 10029

REPORT DATE: October 2014

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2014	Annual	25 Sep 2013 - 24 Sep 2014

4. TITLE AND SUBTITLE
Down-Regulation of Olfactory Receptors in Response to Traumatic Brain Injury Promotes Risk for Alzheimer's Disease

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0582

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Giulio Maria Pasinetti MD., PhD

E-Mail: giulio.pasinetti@mssm.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Icahn School of Medicine at Mount Sinai
1 Gustave L. Levy Place
New York, NY 10029

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Traumatic Brain Injury (TBI) is a risk factor for subsequent development of Alzheimer's disease (AD). Abnormal tau processing is a common pathological feature of TBI and AD and tau neuropathology plays a key role in both TBI complications and AD dementia. This study is based on our recent findings of aberrant down-regulation of specific olfactory receptors (OR) as biological indices for TBI and down-regulation of OR TBI biomarkers following TBI may contribute to TBI-related tau neuropathology. We propose that down-regulation of select OR TBI biomarkers in the brain may contribute to the elevation of tau neuropathological phenotypes, thereby promoting the development of AD dementia among Operation Enduring Freedom (OEF) and Operation Iraqi Freedom (OIF) veterans with exposure to TBI. In Year 1, we found a significant decrease of blood OR contents in a rat model of TBI. We found that activation of OR4M1 by a low affinity ligand resulted in reduced tau phosphorylation via JNK signaling pathway, and a manuscript was published based on this finding. We constructed a virtual 3D structure model for OR4M1 and identified 57 potential ligands for OR activation. In Year 2, we found decreased blood OR content in rats 12 months following blast. We screened the compounds identified from Year 1 in silico screening and confirmed one OR4M1 ligand using cAMP assay. Based on the OR4M1 3D model, we also constructed a 3D model for OR11H1 and performed in silico screening of potential ligands. Outcomes from our study will provide a better understanding of TBI complications and how TBI is related to AD.

15. SUBJECT TERMS
Olfactory receptor; in silico screen; traumatic brain injury

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	11	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

1. INTRODUCTION

Traumatic Brain Injury (TBI) is a risk factor for subsequent development of Alzheimer's disease (AD). Abnormal tau processing is a common pathological feature of TBI and AD and tau neuropathology plays a key role in both TBI complications and AD dementia. This study is based on our recent findings of aberrant down-regulation of specific olfactory receptors (OR) as biological indices for TBI and down-regulation of OR TBI biomarkers following TBI may contribute to TBI-related tau neuropathology. We propose that down-regulation of select OR TBI biomarkers in the brain may contribute to the elevation of tau neuropathological phenotypes, thereby promoting the development of AD dementia among Operation Enduring Freedom (OEF) and Operation Iraqi Freedom (OIF) veterans with exposure to TBI. In Year 1, we found a significant decrease of blood OR contents in a rat model of TBI. We found that activation of OR4M1 by a low affinity ligand resulted in reduced tau phosphorylation via JNK signaling pathway, and a manuscript was published based on this finding. We constructed a virtual 3D structure model for OR4M1 and identified 57 potential ligands for OR activation. In Year 2, we found decreased blood OR content in rats 12 months following blast. We screened the compounds identified from Year 1 in silico screening and confirmed one OR4M1 ligand using cAMP assay. Based on the OR4M1 3D model, we also constructed a 3D model for OR11H1 and performed in silico screening of potential ligands. Outcomes from our study will provide a better understanding of TBI complications and how TBI is related to AD.

2. KEYWORDS

Olfactory receptor; in silico screen; traumatic brain injury

3. ACCOMPLISHMENTS

° **What were the major goals of the project?**

- Milestone 1 (Month 1-24): Based on *in silico* screen of compound libraries and *in vitro* validation, we will identify individual compounds that are able to selectively activate OR with high affinity;
- Milestone 2 (Month 25-30): We will identify cellular signals that are associated with OR-mediated modulation of tau neuropathology;
- Milestone 3 (Month 13-30): We will assess tau neuropathology in a blast model of TBI and whether induction and progression of tau neuropathology might be associated with down- regulation of OR mRNA content.

°What was accomplished under these goals?

Both AD and TBI exhibit tau neuropathology, which plays a key role in TBI complication and AD dementia. Thus, the study was designed to test the relationship between long-term down regulation of ORs and tau-mediated neuropathogenesis. The overall study is separated into three parts: identification of high affinity, selective ligands for select olfactory receptor, *in vitro* study using this high-affinity OR ligands to investigate the impacts of OR downstream signaling on tau neuropathogenic mechanisms and *in vivo* assessment of OR and tau signaling in a rodent model of TBI.

***In vivo* assessment of OR: OR expression in a blast-induced rat model of TBI**

Expression of olfactory receptors in the blood of rats 18 months following blast

In our Year 1 report, we found significant decrease of Olfr735 (rat homolog of OR4X1), Olfr1612 (rat homolog of OR4M1), and Olfr1671 (rat homolog of OR2J3) in the blood 6 months after blast. To assess the long-term effect of blast on the expression of these olfactory receptors, we took blood samples from blasted or control rats 12 months following blast and performed quantitative PCR analysis. We found that the expression levels of these olfactory receptors are still significantly decreased in the blast group compared to the control group 12 months following blast (Figure 1).

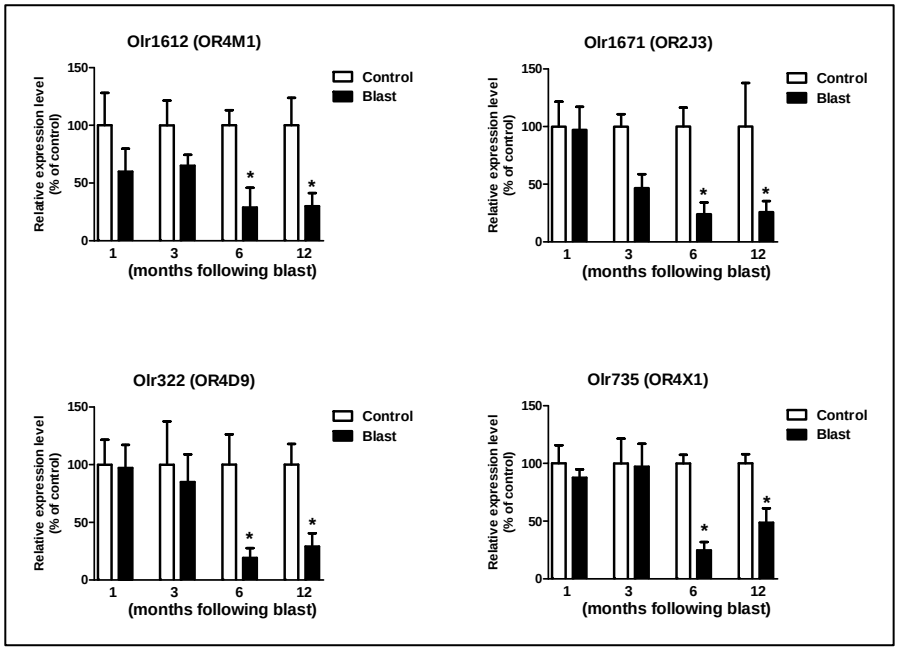

Figure 1: Expression of select olfactory receptors following blast. *RNAs were extracted and first strand cDNA was synthesized using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen). Expression of Olfr322 (rat homolog of OR4D9), Olfr735 (rat homolog of OR4X1), Olfr1612 (rat homolog of OR4M1), and Olfr1671 (rat homolog of OR2J3) were examined by quantitative PCR using Power SYBR Green PCR master mix (Applied BioSystems) on the ABI 7900-HT system (Applied BioSystems). GAPDH were used as internal controls. Data (Mean±SEM) were plotted in GraphPad Prism. $p<0.05$ by 2-tailed t-test.*

To further explore whether the peripheral biomarker level could also be reflected in the brain, we will sacrifice the rats and assess the olfactory receptor expression level in the cortex, hippocampus, and cerebellum by quantitative PCR. We will also assess the tau phosphorylation status in the brain by western blot analysis and immunohistochemistry.

Assessment of candidate OR4M1 ligands for OR4M1-activation activity

In Year 1 report, we summarized outcomes from an *in silico* screening from which we identified a number of candidate ligands for OR4M1. In Year 2, we generated primary cortico-hippocampal neuron culture from NSE-OR4M1 transgenic mice, which we used to test candidates' capability to activate OR4M1. In initial studies, we treated TgOR4M1 cortico-hippocampal neuron culture with individual ligands and assessed for OR4M1-meidated cellular induction of cAMP as an index of OR4M1 activation. In initial studies, we tested 25 candidate OR4M1 ligands at 10 μM. Among the 25 compounds tested, Z221560030 and Z154373220 were able to activate OR4M,1 as indicated by increases in cellular cAMP contents in response to ligand treatment (Figure 2).

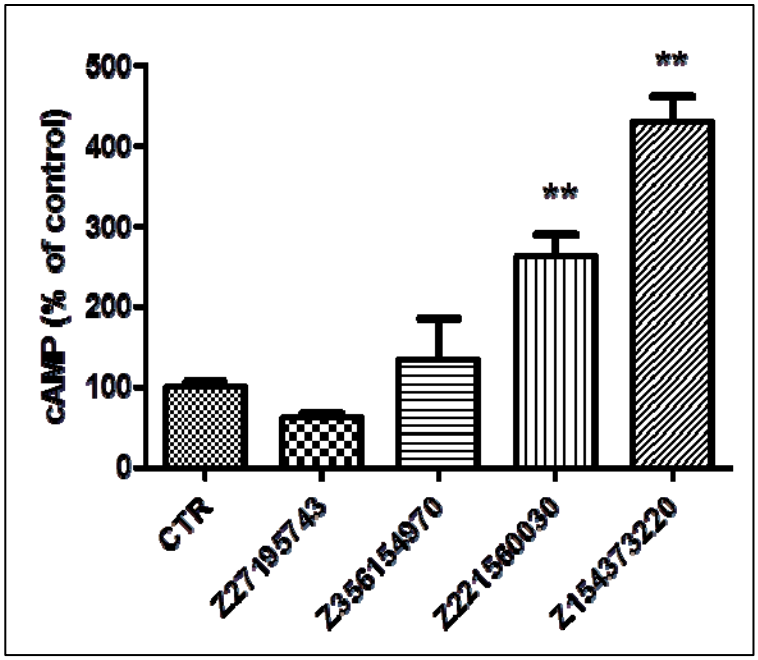

Figure 2: Representative figure of OR4M1 ligand verification by cAMP assay. *Embryonic day 15 cortico-hippocampal neuronal cultures were prepared from TgOR4M1 mice. Cells were seeded onto poly-D-lysine-coated 12-well plates at 5×10^5 cells per well and cultured in Neurobasal medium supplemented with 2% B27, 0.5mM L-glutamine, and 1% penicillin-streptomycin (Life Technologies). On Day 5 of culture, primary cortico-hippocampal neuron cultures were pretreated with 3-isobutyl-l-methylxanthine (IBMX), an inhibitor of cAMP phosphodiesterase, for 10 min, followed by ligand treatment (10μM, all from Enamine) for 10 min. cAMP assay was performed using a colorimetric cAMP ELISA assay kit (Cell Biolabs).*

Both compounds have similar characteristics in that they exhibit a flexible linker region that connects two ring structures (Figure 3A, C). These ring structures occupy the two wider regions in the OR4M1 pocket, while the linker occupies the narrow connecting region (Figure 3B, D).

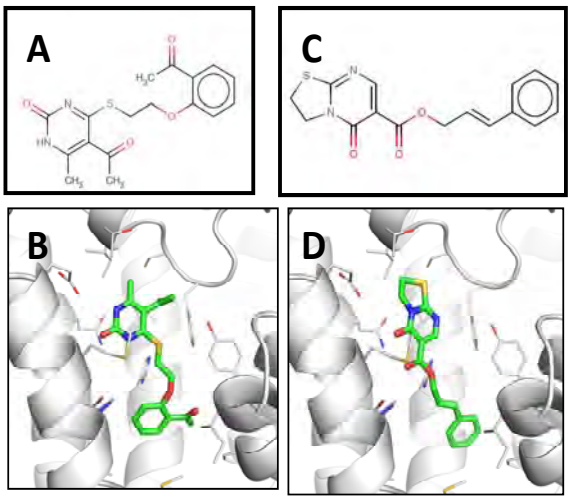

Figure 3: Structures of the two experimentally validated hits for OR4M1 and docking of two positive ligands in the OR4M1 binding pocket. *(A)Structure of Z221560030; (B) Predicted binding pose for Z221560030 in the binding site of the OR4M1 model; (C) Structure of Z154373220; (D) Predicted binding pose for Z154373220 in the binding site of the OR4M1 model*

We continued to test the dose-responsiveness of OR4M1 activating ligands to activate cellular OR4M1 in E15 cortico-hippocampal cultures generated from OR4M1 transgenic mice. Using the cAMP assay, we found that the activation of OR4M1 by Z221560030 reached peak activity at 100 nM (Figure 4). In future studies, we will use this concentration of Z221560030 to treat TgOR4M1 primary neuron cultures in order to assess tau phosphorylation and signaling.

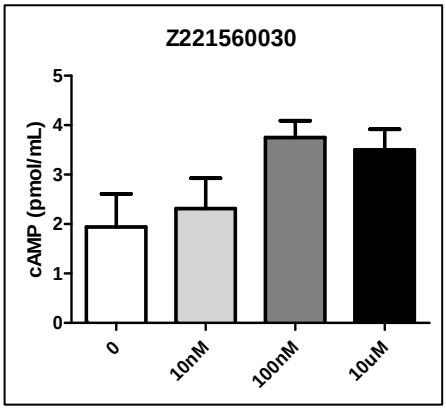

Figure 4: Activation of OR4M1 by Z221560030. *Embryonic day 15 cortico-hippocampal neuronal cultures were prepared from TgOR4M1 mice. Cells were seeded onto poly-D-lysine-coated 12-well plates at 5×10^5 cells per well and cultured in Neurobasal medium supplemented with 2% B27, 0.5mM L-glutamine, and 1% penicillin-streptomycin (Life Technologies). On Day 5 of culture, primary cortico-hippocampal neuron cultures were pretreated with 3-isobutyl-l-methylxanthine (IBMX), an inhibitor of cAMP phosphodiesterase, for 10 min, followed by Z221560030 treatment (10nM, 100nM and 10µM, from Enamine) for 10 min. cAMP assay was performed using a colorimetric cAMP ELISA assay kit (Cell Biolabs).*

Based on information we generated relating to chemical structures of the small molecule ligands tested and OR4M1-activating activities, we optimized our *in silico* screening model system and conducted a second round of *in silico* screening in order to identify candidate OR4M1 ligands with improved affinity and specificity for OR4M1. The strategy to obtain preliminary Structure--Activity Relationship (SAR) data before attempting the synthesis of new compounds involves the selection of commercially available compounds that modify substructures of the two hits to identify their role in the activity of the molecule, and possibly provide more potent compounds. Structural analogs that conserve at least one region of the original hits were selected from the ZINC database, as well as from our in-house FSL library of drug-like compounds and the NCI Open library of compounds. For both hits, the structurally similar compounds explore the role of the two ring structures at each end of the linker, and possible variations that may improve potency. The FSL library contains 9 compounds that mimic some aspects of Z221560030 and may provide SAR data and possibly more potent compounds. The ZINC library contained an additional 55 compounds for Z221560030 SAR. We will next perform bioactivity assays to test their potential in activating OR4M1.

***In Silico* Modeling of OR11H1**

A model of the 3D structure of olfactory receptor OR11H1 was built by comparative modeling based on the previously modeled OR4M1 structure. The model of OR11H1 was built using MODELLER version 9.12, using the OR4M1/OR11H1 sequence alignment and the OR4M1 model as input. As expected, due to the similarity

between OR4M1 and OR11H1, predicted olfactory receptor binding site residues are spatially close in the OR11H1 model, suggesting that the alignment is reliable. Loop regions close to the active site were further refined using the loop modeling routine in MODELLER. A total of 100,000 conformations for the loop were generated, and the best scoring one, based on the DOPE potential, was selected as the final model. Using program SiteHound on the final model, a binding pocket is clearly identified (Figure 5). The binding pocket is in the same location as the binding pocket in OR4M1, but it is distinct in terms of shape, volume, and residues facing into the cavity. The OR11H1 pocket contains two regions separated by a narrow "neck". Overall, the OR11H1 pocket is more constrained than the OR4M1 pocket.

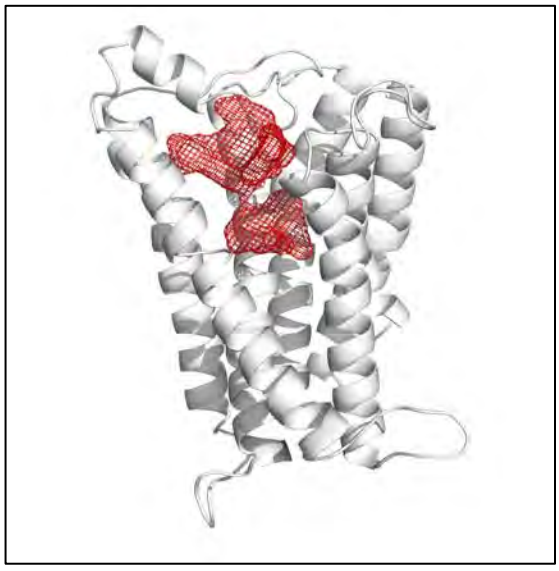

Figure 5: Structural model of OR11H1 and predicted binding pocket. *The predicted binding pocket identified by SiteHound* is shown as a red mesh.

Virtual Screening of the OR11H1 binding pocket

The pocket identified in the OR11H1 model was used as a target for virtual screening of small molecule compounds. A set of ~5 million lead-like compounds derived from the ZINC library of commercially available compounds was docked into the binding site using program DOCK version 6.5. Lead-like compounds were selected to facilitate optimization of validated hits. The top 500 hits from the DOCK screening (ranked by docking score) were visually inspected, and 83 compounds, which showed good occupancy of the predicted binding pocket and at least two hydrogen bonds with the protein, were selected for experimental validation. The selected compounds were clustered based on similarity, as measured by the Tanimoto coefficient. Single-linkage clustering was used, with a Tanimoto coefficient of 0.7 as the cutoff. This resulted in 7 clusters of structurally similar compounds (containing a total of 19 compounds) and 64 compounds that did not fall into clusters. The lower level of clustering compared to the OR4M1 virtual screening hits might be explained by the fact that the OR11H1 binding pocket has two distinct regions. Several of the hits occupy only the lower part of the binding pocket, while other compounds occupy both the top and bottom regions (Figure 6).

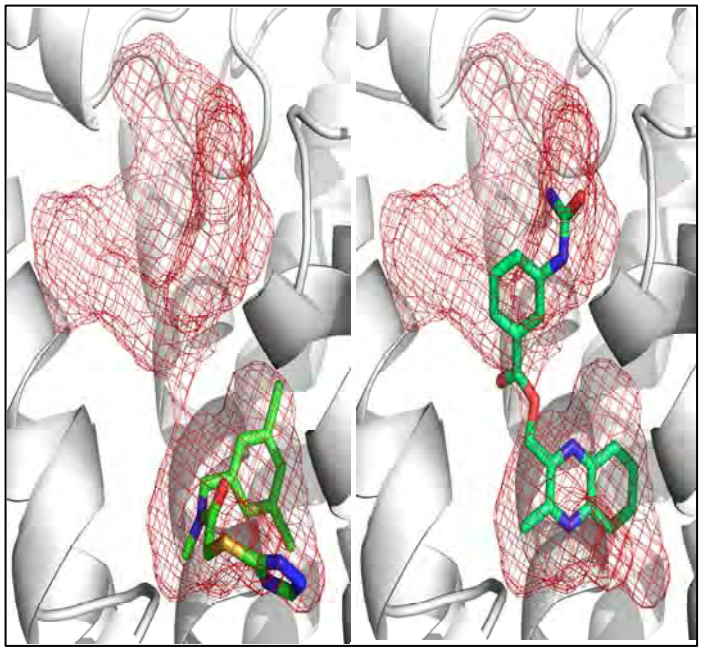

Figure 6: Example of virtual screening hits. ***(Left)*** *One of the top scoring compounds (green) occupying only the lower part of the OR11H1pocket (red mesh).* ***(Right)*** *One of the top scoring compounds (green) occupies the top and bottom parts of the OR11H1 pocket.*

The screening results show higher consistency when the binding modes are compared, with compounds in different clusters and even un-clustered compounds showing similar binding modes, especially in the lower part of the binding pocket (Figure 7).

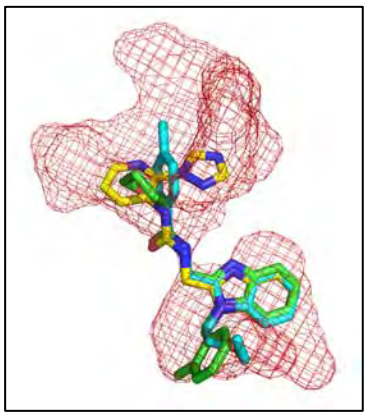

Figure 7: Structurally dissimilar compounds show similar binding mode. *Representative compounds from cluster 1, cluster 2, and one un-clustered compound are shown. The binding mode is highly similar in the lower pocket and "neck" region of the OR11H1 pocket.*

In Year 2 studies, we found a significant decrease of blood OR content in a rat model of TBI 12 months after blast. We tested the compounds identified from Year 1 *in silico* screening for their ability to activate OR4M1 *in vitro*, and found 2 positive ligands. We performed another round of *in silico* screening based on the structures of the two positive ligands, and we identified structural analogs. We constructed a virtual 3D structural model for OR11H1 to screen for potential high-affinity ligands. The pocket identified in the OR11H1 model was used as a target for virtual, high-throughput screening of small molecule compounds. 83 compounds were identified. Among these 83 compounds, 19 compounds were clustered into 7 clusters of structurally similar compounds, and 64 compounds did not fall into clusters. In ongoing studies, we will screen the identified structural analogs and use high-affinity ligands to assess downstream tau signaling. We will also assess the brain OR content and tau phosphorylation in this rat model of TBI. Outcomes from our study will provide a better understanding of TBI complications and how these complications are related to AD.

What opportunities for training and professional development has the project provided?
Nothing to report

How were the results disseminated to communities of interest?
Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?
In ongoing studies, we will screen the identified structural analogs and use high-affinity ligands to assess downstream tau signaling. We will also assess the brain OR content and tau phosphorylation in this rat model of TBI. Outcomes from our study will provide a better understanding of TBI complications and how these complications are related to AD.

4. IMPACT

° **What was the impact on the development of the principal discipline(s) of the project?**

- We assessed blood OR content in a rat model of TBI 12 months after blast. We found significant decrease of select olfactory receptors and established a cause-and-effect relationship between TBI exposure and long-term down-regulation of select ORs.
- We screened compounds identified from Year 1 *in silico* screening for their ability to activate OR4M1, and we tested dose-response of positive ligands.
- We performed a second round of *in silico* screening and identified structural analogs of positive ligands.
- We constructed a virtual 3D structure for OR11H1.
- The pocket identified in the OR11H1 model was used as a target for virtual screening of small molecule compounds. 83 compounds were identified. Among these 83 compounds, 19 compounds were clustered into 7 clusters of structurally similar compounds, and 64 compounds did not fall into clusters.

°What was the impact on other disciplines?
Nothing to report

°What was the impact on technology transfer?
Nothing to Report

°What was the impact on society beyond science and technology?
Nothing to Report

5. CHANGES/PROBLEMS

- **Changes in approach and reasons for change**
  Nothing to Report

- **Actual or anticipated problems or delays and actions or plans to resolve them**
  Nothing to Report

- **Changes that had a significant impact on expenditures**
  Nothing to Report

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
  Nothing to Report

- **Significant changes in use or care of human subjects**
  Nothing to Report

- **Significant changes in use or care of vertebrate animals.**
  Nothing to Report

- **Significant changes in use of biohazards and/or select agents**
  Nothing to Report

6. PRODUCTS

°Publications, conference papers, and presentations

- **Journal publications**
  Nothing to Report
- **Books or other non-periodical, one-time publications**
  Nothing to Report
- **Other publications, conference papers, and presentations**
  Lap Ho, Wei Zhao, Roberto Sanchez, Merina Varghese, Daniel Freire, Giulio Maria Pasinetti, ***Activation of ectopically expressed olfactory receptors in the brain attenuates tau-processing in response to mild traumatic brain injury****, AAIC, Copenhagen, Denmark, 2014*

°Website(s) or other Internet site(s)
Nothing to Report.

°Technologies or techniques
Nothing to Report

°Inventions, patent applications, and/or licenses
Nothing to Report

°Other Products
OR11H1 virtual 3D model
OR4M1 transgenic mouse

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Giulio M. Pasinetti*
Project Role:	*Principal Investigator*
	No change
Name:	*Wei Zhao*
Project Role:	*Assistant Professor*
	No change
Name:	*Lap Ho*
	Associate Professor
	No Change

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  Nothing to Report

- **What other organizations were involved as partners?**
  Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:**
  N/A

- **QUAD CHARTS:**
  N/A

9. APPENDICES

Nothing to Report

REFERENCES

Elder GA, Dorr NP, De GR, Gama Sosa MA, Shaughness MC, Maudlin-Jeronimo E, Hall AA, McCarron RM, Ahlers ST (2012) Blast exposure induces post-traumatic stress disorder-related traits in a rat model of mild traumatic brain injury. J Neurotrauma 29:2564-2575.

Eswar N, John B, Mirkovic N, Fiser A, Ilyin VA, Pieper U, Stuart AC, Marti-Renom MA, Madhusudhan MS, Yerkovich B, Sali A (2003) Tools for comparative protein structure modeling and analysis. Nucleic Acids Res 31:3375-3380.

Fiser A, Do RK, Sali A (2000) Modeling of loops in protein structures. Protein Sci 9:1753-1773.
Ghersi D, Sanchez R (2009) EasyMIFS and SiteHound: a toolkit for the identification of ligand-binding sites in protein structures. Bioinformatics 25:3185-3186.

Irwin JJ, Shoichet BK (2005) ZINC--a free database of commercially available compounds for virtual screening. J Chem Inf Model 45:177-182.

Johnson VE, Stewart W, Smith DH (2012). Widespread tau and amyloid-beta pathology many years after a single traumatic brain injury in humans, Brain Pathol. 22(2), 142-9.

Man O, Gilad Y, Lancet D (2004) Prediction of the odorant binding site of olfactory receptor proteins by human-mouse comparisons. Protein Sci 13:240-254.

Marti-Renom MA, Madhusudhan MS, Sali A (2004) Alignment of protein sequences by their profiles. Protein Sci 13:1071-1087.

Moustakas DT, Lang PT, Pegg S, Pettersen E, Kuntz ID, Brooijmans N, Rizzo RC (2006) Development and validation of a modular, extensible docking program: DOCK 5. J Comput Aided Mol Des 20:601-619.

Okada T, Sugihara M, Bondar AN, Elstner M, Entel P, Buss V (2004) The retinal conformation and its environment in rhodopsin in light of a new 2.2 A crystal structure. J Mol Biol 342:571-583.

Shen MY, Sali A (2006) Statistical potential for assessment and prediction of protein structures. Protein Sci 15:2507-2524.

Sivananda, TM, and Thakur MK (2012). Traumatic brain injury: a risk factor for Alzheimer's disease, Neurosci. Biobehav. Rev. 36(5), 1376-81.

Teague SJ, Davis AM, Leeson PD, Oprea T (1999) The Design of Leadlike Combinatorial Libraries. Angew Chem Int Ed Engl 38:3743-3748.

Van Den HC, Thornton, E, Vink, R. (2007) Traumatic brain injury and Alzheimer's disease: a review. Prog. Brain Res. 161, 303-16.

Zhao W, Ho L, Varghese M, Yemul S, Dams-O'Connor K, Gordon W, Knable L, Freire D, Haroutunian V, Pasinetti GM (2013) Decreased level of olfactory receptors in blood cells following traumatic brain injury and potential association with tauopathy. J Alzheimers Dis 34:417-429.